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DESCRIPTION

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A METHOD FOR INDUCING IMMUNOSUPPRESSIVE CELLS AND  
A CULTURE DEVICE TO BE USED THEREFOR

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TECHNICAL FIELD

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CI

The present invention relates to a method for inducing immunosuppressive cells and a culture device to be used therefor. More particularly, the present invention relates to a method for inducing immunosuppressive cells by culturing human cells with the use of a culture device having an affinity for protein, and the culture device to be used therefor.

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BACKGROUND ART

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Autoimmune diseases and rejection in transplantation are caused by the following: host cells recognize self-cells or an organ transplanted as a foreign body, and destroy the self-cells or the organ transplanted. Some cases of those diseases show that they are caused by abnormality of immunosuppressive cells in quantity or quality (see Macintosh and Drachman, Science vol.232, p.401(1986)). Balashov et al. report that MS (multiple sclerosis) patients show remarkable decrease in efficiency for inducing immunosuppressive T cells due to an autologous mixed lymphocyte reaction and such phenomenon is a cause of an attack of the MS disease (see Balashov et al., Journal of Clinical Investigation, vol.95, p.2711(1995)). Accordingly, it is considered that the recovery of the immunosuppressive cells of such patients may be

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effective in a therapeutic method for such patients. Thus for several examples of inducing immunosuppressive T cells in a system using animals such as mice are reported by, for example, Fresno et al. (see Fresno et al., Journal of Experimental Medicine, 5 vol.163, p.1246 (1980)). Further, as an example of inducing the immunosuppressive cells in human, there is a method ~~with~~ utilizing the autologous mixed lymphocyte reaction described in the above reference which has <sup>been</sup> recently reported. However, this method requires the <sup>treatment</sup> ~~addition~~ of a culture medium of human cells <sup>adding a plurality</sup> ~~or~~ <sup>plurality</sup> of cytokines to <sup>the</sup> ~~a~~ culture. Thus, the method is complicated in operation. Moreover, since the above-mentioned method is suspension culture system, foreign bodies such as lectins (PHA or ConA) used in treatment adhere to the cells. Since those foreign 15 bodies can not be removed completely from the cells by washing, the above-mentioned method still has the possibility of foreign <sup>body</sup> ~~bodies~~ contamination. Accordingly, the <sup>method</sup> ~~method~~ <sup>it would</sup> never be considered to be desirable and <sup>thus</sup> ~~then~~ <sup>it would</sup> can not be utilized in treatment that targets induction of immunosuppressive cells 20 outside of the body of a patient. The present invention provides an efficient therapeutic system for autoimmune diseases which includes the steps of: taking blood cells out from a patient; effectively inducing immunosuppressive cells, especially immunosuppressive T cells using an immobilized-protein system; 25 and returning the resulting blood cells to the patient.

## DISCLOSURE OF THE INVENTION

The present inventors have studied to improve a technique for preparing cells which selectively suppress hypersensitivity of the immune system which causes autoimmune diseases such as <sup>those</sup> ~~the~~ mentioned above, especially immunosuppressive T cells and have completed the present invention.

The present invention provides a method for inducing immunosuppressive cells by culturing human cells with the use of  
10 (1) a culture device having an affinity for protein, being previously coated with a protein such as an antibody or a cytokine, or without  
B any coating, or (2) <sup>a</sup> ~~an~~ culture device having an affinity for protein, placing a protein such as the antibody or the cytokine in the device together with the human cells; and the culture device to be used  
15 therefor.

That is, the present invention relates to a method for inducing immunosuppressive cells, which comprises culturing human cells with the use of a culture device having an affinity for  
B protein ~~(Claim 1)~~.

20 Further, the present invention relates to the inducing  
B method of ~~Claim 1~~, wherein the culture device is previously coated with one or more cytokines or antibodies against surface antigens (Claim 2).

Moreover, the present invention relates to the inducing  
25 B method of ~~Claim 1~~, wherein the culture device is previously coated with two or more antibodies against surface antigens, each of said  
B antibodies recognizing different epitope ~~(Claim 3)~~.

Further, the present invention relates to the inducing  
B method of ~~Claim 1~~, wherein human cells and one or more  
B cytokines or antibodies against surface antigens are mixed (~~Claim~~  
B 4).

5 Furthermore, the present invention relates to the  
B inducing method of ~~Claim 1~~, wherein human cells and two or more  
antibodies against surface antigens, each of said antibodies  
B recognizing different epitope, are mixed (~~Claim 5~~).

10 Moreover, the present invention relates to the inducing  
method of ~~Claim 2, 3, 4 or 5~~, wherein the antibody against surface  
B antigens is an anti-CD2 antibody or an anti-CD3 antibody (~~Claim~~  
B 6).

15 Further, the present invention relates to the inducing  
B method according to ~~Claim 2, 3, 4, 5 or 6~~, wherein the antibody  
against surface antigens is an anti-CD2 antibody, said anti-CD2  
antibody binding to a site of CD2 that participates in the binding  
B of LFA-3 to CD2 (~~Claim 7~~).

20 Further, the present invention relates to the inducing  
B method of ~~Claim 2, 3, 4, 5 or 6~~, wherein the antibody against  
surface antigens is an anti-CD2 antibody, said anti-CD2 antibody  
binding to a site of CD2 other than a site of CD2 which  
B participates in the binding of LFA-3 to CD2 (~~Claim 8~~).

25 Moreover, the present invention relates to the inducing  
B method of ~~Claim 3 or 5~~, wherein two or more antibodies against  
surface antigens are anti-CD2 antibodies comprising a  
combination of one or more antibodies which bind to a site of CD2  
that participates in the binding to LFA-3 to CD2 and one or more

antibodies which bind to a site of CD2 other than the site of CD2

*B* which participates in the binding of LFA-3 to CD2 ~~(Claim 9).~~

Further, the present invention relates to the inducing

*B* method of ~~Claim 2, 3, 4, 5, 6, 7 or 8~~, wherein one antibody against

*B* surface antigens is the anti-CD2 antibody TS2/18 ~~(Claim 10).~~

Moreover, the present invention relates to the inducing

*B* method of ~~Claim 2, 3, 4, 5, 6, 7 or 8~~, wherein one antibody against

surface antigens is the F(ab)<sub>2</sub> fragment of the anti-CD2 antibody

*B* TS2/18 ~~(Claim 11).~~

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Further, the present invention relates to the inducing

*B* method of ~~Claim 2 or 4~~, wherein the cytokine is IL-2 or GM-CSF

*B* ~~(Claim 12).~~

Moreover, the present invention relates to the inducing

method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12~~, wherein 0.5

15 to 10 % by volume of serum based on a culture medium is mixed

*B* in the culture ~~(Claim 13).~~

Further, the present invention relates to the inducing

*B* method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12~~, wherein no

*B* serum is mixed with the culture medium in the culture ~~(Claim 14).~~

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Moreover, the present invention relates to the inducing

*B* method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14,~~

*B* wherein a term of the culture ranges between 1 and 7 days ~~(Claim~~

*B* ~~15).~~

Further, the present invention relates to the inducing

25 method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15,~~

wherein the culture device is made of material having an affinity

for protein ~~(Claim 16).~~

Moreover, the present invention relates to the inducing  
B method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or~~  
B-16, wherein culture device is made of plastic material (~~Claim 17~~).

Further, the present invention relates to the inducing  
5 B method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or~~  
B-16, wherein the culture device is made of glass material (~~Claim 18~~).

Moreover, the present invention relates to the inducing  
B method of ~~Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,~~  
B-17 or 18, wherein the culture device is a closed plane plate vessel  
10 B or a closed vessel charged with spherical fine-particles (~~Claim 19~~).

Further, the present invention relates to culture device  
having an affinity for protein, which is used for inducing  
B immunosuppressive cells by culturing human cells (~~Claim 20~~).

Moreover, the present invention relates to the culture  
15 B device of ~~Claim 20~~, wherein the device is previously coated with  
one or more cytokines or antibodies against surface antigens  
B (~~Claim 21~~).

Further, the present invention relates to the culture  
B device of ~~Claim 20~~, wherein the device is previously coated with  
20 two or more antibodies against surface antigens, each of said  
B antibodies recognizing different epitope (~~Claim 22~~).

Moreover, the present invention relates to the culture  
B device of ~~Claim 21 or 22~~, wherein the antibody against surface  
antigens is anti-CD2 antibody or anti-CD3 antibody (~~Claim 23~~).

25 Further, the present invention relates to the culture  
device of ~~Claim 22 or 23~~, wherein one or more antibodies that  
recognize different epitope are the anti-CD2 antibody TS2/18

~~(Claim 24)~~

## BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is an explanatory view of one embodiment of the culture device of the present invention; (a) being a sectional explanatory view of the device when human cells are placed in the device; (b) being a sectional explanatory view of the device (a) being rotated in 90 degrees; and (c) being a top explanatory view of (b).

10 Figure 2 is a sectional explanatory view of another embodiment of the culture device of the present invention.

Figure 3 is a graph that shows the immunosuppressive effect of immunosuppressive cells obtained by the present invention ((a) of EXAMPLE 1) on T cell activation reaction.

15 Figure 4 is a graph that shows the immunosuppressive effect of immunosuppressive cells obtained by the present invention ((b) of EXAMPLE 1) on T cell activation reaction.

Figure 5 is a graph that shows the immunosuppressive effect of immunosuppressive cells obtained by the present invention ((c) of EXAMPLE 1) on T cell activation reaction.

20 Figure 6 is a graph that shows the immunosuppressive effect of immunosuppressive cells obtained by the present invention ((d) to (f) of EXAMPLE 1) on T cell activation reaction.

25 Figure 7 is a graph that shows the immunosuppressive effect of immunosuppressive cells obtained by the present invention (EXAMPLE 2) on T cell activation reaction.

## BEST MODE FOR CARRYING OUT THE INVENTION

The expression "having an affinity for protein" means having the property that a protein can be immobilized by a bond such as hydrophobic bond, covalent bond or ionic bond.

5       The culture device of the present invention may be material having an affinity for protein, being non-cytotoxic and being sterilizable. Preferably the device is made of material having an affinity for protein and the material is preferably plastic material or glass material. Plastic material means thermosetting  
10 resin or thermoplastic resin. Polymer is more preferable because of its good moldability. Polystyrene is particularly preferable because polystyrene has high affinity for protein and is colorless and transparent. Glass material means silicate, borate, phosphate and the like which solidify without forming crystals  
15 (vitrification). Silicate glass is more preferable because of a strong vitrification tendency. Crystallized glass which is formed by heat treatment of silicate glass is particularly preferable because it has a good moldability and good impact resistance. In order to confer an affinity for protein on the culture device, the material may be  
20 treated for increasing adsorbability of protein. The usual culture device is coated with protein such as collagen so that the adsorbability of protein is decreased. This is not preferable for the device of the present invention. In the culture device of the present invention, the shape of the material to which cells directly  
25 attach is preferably plane shape or spherical shape, because it is necessary to immobilize protein uniformly and further it is necessary that the cells can uniformly and efficiently adsorb



thereto. More preferably the culture device is a closed plane plate vessel or a closed vessel charged with spherical fine-particles. As the plane vessel, a usual culture flask, a culture dish, a vessel with a culture plate-like shape, or multistage plate as shown in  
5 Figure 1(a) to 1(c) can be used.

The multistage plate as shown in Figure 1 is an embodiment of the culture device of the present invention. As shown in Figure 1(a), a cell suspension (2) containing human cells (1) is placed in the plate from the direction indicated by the arrow.  
10 As shown in Figure 1(b), in the culture the device of Figure 1(a) is rotated in 90-degrees. During culture, 5 % CO<sub>2</sub> is passed through the breathable filter (3). The immunosuppressive cells induced can be released by somewhat strongly shaking of the vessel and recovered from the cell recovery opening (4). The  
15 Figure 1(c) is a top view of Figure 1(b). When such multistage plate is employed, it has the advantages that it can treat many cells at the same time and it can induce plural types of cells having different functions at the same time. For example, by changing the antibody employed for coating, both  
20 immunosuppressive cells and cells with ability for activating an immunosuppressive mechanism can be simultaneously induced. When the material of the device is spherical, fine-particles (small spherical beads) can be used in a vessel as shown in Figure 2.

The vessel shown in Figure 2 is another embodiment of  
25 the present invention. In Figure 2, 5 % CO<sub>2</sub> can be fed to the cell suspension (22) including human cells through the breathable filter (23). The spherical fine-particles (25) are present in the cell

suspension (22) and adsorb the human cells. The immunosuppressive cells induced are recovered in the adsorbing form on the spherical fine-particles by opening the <sup>stopcock</sup>cock (26). The number (27) shows a side wall. And then, the cells induced can be recovered from the fine-particles by washing the fine-particles with, for example, saline.

In the present invention, a plane plastic plate that is mainly used in ELISA reactions is particularly preferably employed as the device.

10 The term "human cells" means cells that constitute the human body and cells derived from such cells. As the human cells of the invention, there can be employed, for instance, human peripheral blood cells, human peritoneal cells, human thymocytes, human bone marrow cells, human established T cells and the like.

15 In case that a patient is actually treated by a method wherein cells are entered into the patient, it is most preferable to use the patient's cells in view of immunological rejection or immunosuppression effect. Among the patient's cells, human peripheral blood cells of the patient are preferable because they are easily

20 collected. Human peripheral blood cells can be prepared from the following: venous blood collected from human is layered on Ficoll (Ficoll-paque, made by Pharmacia Biotech) in a 50 ml polystyrene tube and centrifuged for 20 minutes at 1500 revolutions per

25 <sup>rpm</sup>minute, and then, a lymphocyte fraction and a monocyte fraction that are formed in a boundary surface are recovered and are washed with RPMI-1640 medium in 3 times (Ficoll centrifugation method). The resulting human peripheral blood cells are

inoculated into a culture medium and cultured overnight, and suspension cells are collected to give T cells.

The term "antibody against surface antigens" means an antibody against a protein that mediates intercellular adhesion; an antibody against a protein that, in adhesion, secondarily participates in binding to a molecule on the other cell; and an antibody against a receptor for various cytokines. As the antibody, there can be employed, for instance, an antibody against the immunoglobulin superfamily, an antibody against the integrin superfamily, an antibody against cadherin or an antibody against IL-2 receptor. Among them, the antibody against the immunoglobulin superfamily is preferable, and an antibody having an affinity for CD2 antigen or CD3 antigen of the human T cells or analog thereof is particularly preferable.

The expression "two or more antibodies against surface antigens, each of the antibodies recognizing different epitope" means two or more antibodies that <sup>each recognize a</sup> ~~recognize each~~ different site of a surface antigen molecule as an antigen and bind to each different site. The kind of antibodies is preferably two. As a concrete examples of the antibodies, there can be preferably employed, for instance, two antibodies which recognize CD2 molecule and recognize different epitope. A combination of the anti-CD2 antibody TS2/18 and an antibody which is produced from the hybridoma S33H2NO10 can more preferably be used (see the following REFERENCE EXAMPLE 2, EXAMPLE 2 and TEST EXAMPLE 2). The ratio of the combination of those antibodies is preferably 1 : 9 to 9 : 1 (ratio by weight), particularly preferably

1 : 1 (equivalent weight).

As the "antibody against CD2 antigen (anti-CD2 antibody)", there can be employed, for instance, a monoclonal antibody against each of various epitopes of CD2 or a polyclonal antibody against CD2 molecule or the like. A F(ab)<sub>2</sub> fragment of  
5 such antibody can also be employed. Among them, the anti-CD2 antibody TS2/18 and the F(ab)<sub>2</sub> fragment thereof are more preferable because they have a strong affinity for CD2.

The "anti-CD2 antibody that can bind to a site of CD2  
10 that participates in the binding of LFA-3 to CD2", which is a preferable antibody against surface antigens, means an anti-CD2 antibody with ability for inhibiting the binding of LFA-3 molecule to CD2 molecule. Concretely this type of antibody is judged by  
15 measuring as to whether the binding of sheep erythrocyte to a human T cell strain (rosette formation reaction; see Current Protocols in Immunology, vol.1, section 7.2.1 (1991)) such as human peripheral blood T cells or Jurkat cells (see U. Schnider et al., Int. J. Cancer, vol.19, p.621 (1977)), is inhibited.

The "anti-CD2 antibody that binds to a site of CD2 other  
20 than a site of CD2 which participates in binding of LFA-3 to CD2", which is a preferable antibody against surface antigens, means an antibody that does not have the ability for inhibiting rosette formation.

As the "antibody against CD3 antigen (anti-CD3  
25 antibody)", there can be employed, for instance, a monoclonal antibody, a polyclonal antibody or a F(ab)<sub>2</sub> fragment thereof that can recognize CD3 molecule. Among them, the anti-CD3 antibody

(OKT3) is more preferable because it has a strong affinity for CD3 and T cell-activating ability.

As the "cytokine", there can be employed, for instance, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-8 receptor family, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, GM-CSF, G-CSF, TGF- $\beta$ , TNF- $\alpha$ , TNF- $\beta$ , IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , a macrophage activating factor, a macrophage migration inhibition factor or the like. Especially, IL-2 and GM-CSF are preferable because they are effective in growth of T cells and production of cytokines. Those cytokines can be used alone or in combination thereof. IL-2 and GM-CSF may be isolated from peripheral blood or prepared from a gene recombination method.

The term "immunosuppressive cells" means cells that have the ability to inhibit immune reaction. The measurement of the ability to inhibit immune reaction can be carried out by using a reaction system in which peripheral blood cells prepared from two or more different individuals are mixed (MLR) or a reaction system in which peripheral blood cells from an individual who has T cells (memory T cells) being responsive to a certain antigen, and the antigen are mixed and the like. As explained in the following TEST EXAMPLES 1 and 2, in vitro, cells probably having immunosuppressive activity are mixed with human peripheral blood lymphocytes (hereinafter also referred to as PBL) that is newly obtained from the same person. With the resulting mixture, is mixed PPD (Purified protein derivative; made by Japan B.C.G. Seizo Co.,Ltd.) and cultured under the condition at 37°C with 5 % CO<sub>2</sub>, for several days, followed by mixing of [<sup>3</sup>H]-thymidine. After

culture at 37°C for an additional 5 to 16 hours, the radioactivity in the cells is counted with a scintillation counter, thereby measuring the ability for inhibiting immune reaction.

Immunosuppressive cells can be obtained by culturing human cells in the culture device or in the culture device to which the antibody against surface antigens and/or the cytokine is immobilized or not immobilized; or immunosuppressive cells can be obtained by culturing human cells in a system where the antibody against surface antigens and/or the cytokine is mixed with the culture medium at the same time, or before or after. In case that human cells are placed in the device at first, a short time for the mixing of the antibody and/or the cytokine (within 3 days) is preferable. More preferably, human cells are placed later (within one day) or at the same time. Particularly preferably, the human cells are placed at the same time. The amount of those protein to be immobilized or placed depends on the protein to be used, and therefore, there is no limitation in particular. The preferable amount ranges between 0.1 and 10  $\mu$ g/ml against the culture medium. When the amount is too little, induction of immunosuppressive activity may not occur and when the amount is in excess, the cells may be damaged.

As the culture medium, there can be employed, for instance, a culture medium for animal cells, D-MEM or RPMI-1640. Serum may not be mixed or may be mixed with the medium. When serum is mixed, autologous serum is preferably employed. Autologous serum means a serum obtained from the same individual from which the human cells to be cultured are prepared.

B Autologous serum is the most preferable because cell growth rate is good and non-specific immunoreaction <sup>rarely occurs</sup> ~~is hardly occurred~~ when autologous serum is used. The amount of serum to be mixed is not limited in particular. Generally, the amount ranges between 5 0.5 and 10 % by volume (V/V %). Immunosuppressive cells can be obtained by culturing human cells in the above-mentioned culture medium, usually under the condition at 37°C with 5 % CO<sub>2</sub>, preferably for 1 to 7 days, more preferably for 1 to 5 days, most preferably for 1 to 3 days.

10 Since the immunosuppressive cells are induced in the immobilized system, the immunosuppressive cells can be released and recovered by the usual method; for example, by somewhat strongly shaking the culture device.

15 There is no limitation as to a dosage route for the immunosuppressive cells induced by the method of the present invention; systematic or local administration using medical device such as a catheter is preferable.

20 The amount of cells to be administered depends on purposes of administration such as the kind of disease and an extent of symptoms of the patient to be administered. They are administered from 10<sup>6</sup> to 5×10<sup>8</sup> cells for an adult in a daily dose, preferably from 10<sup>6</sup> to 10<sup>8</sup> cells, most preferably from 10<sup>6</sup> cells.

25 The present invention provides a simple and efficient method for inducing immunosuppressive cells and also provides a therapeutic method for treating various autoimmune diseases and for suppression of rejection to an organ transplanted.

The present invention will be more specifically described

and explained by means of the following EXAMPLES. The present invention is not limited to EXAMPLES.

#### REFERENCE EXAMPLE 1

5 Preparation of F(ab)<sub>2</sub> fragments of the anti-CD2 antibody  
TS2/18

One ml of the antibody solution including 10 mg/ml (PBS) of TS2/18 (American Type Culture Collection (ATCC): HB195) was dialyzed against 200 ml of 20 mM acetate buffer, pH4.5, overnight. After the dialysis, the antibody sample was mixed with 0.25 ml of pepsin-immobilized carrier (made by Pierce) previously equilibrated with 20 mM acetate buffer, followed by incubation with strongly shaking at 37°C for 4 hours. The reaction was stopped by mixing 1.5 ml of 10 mM Tris-HCl, pH7.5. The resulting F(ab)<sub>2</sub> fragments were isolated using a gel filtration column (Column : Sephacryl S-200, Pharmacia Biotech; Solvent : PBS, Flow Rate : 0.6 ml/min).

#### EXAMPLE 1

20 Preparation of immunosuppressive cells by culturing human peripheral blood cells using the culture device made of plastic plate

Using RPMI-1640 (made by Sigma) including 10 %(v/v) (hereinafter referred to as 10 %) autologous serum  $1.5 \times 10^6$  cells/ml of a normal human peripheral blood cell suspension was prepared from peripheral blood collected from a normal human.

Each of 100 <sup>aliquots</sup> ~~μl~~ of the cell suspension <sup>were</sup> ~~was~~ seeded <sup>in</sup> ~~to~~ a total 20



5 wells in a 96-wells ELISA plate (made by Corning Inc.) and the culture was started (a). Each of 20 wells in 96-wells ELISA plates was previously coated with GM-CSF (made by RD Systems) and IL-2 (made by Becton Dickinson Immunocytometry Systems) in a concentration of 10 ng/ml (PBS) and 20 U/ml (PBS), respectively (b); coated with 10  $\mu$ g/ml (PBS) of F(ab)<sub>2</sub> fragments of the anti-CD2 antibody TS2/18 prepared in REFERENCE EXAMPLE 1 (c); coated with 10  $\mu$ g/ml (PBS) of the anti-CD3 antibody (OKT3) (made by Orth Diagnostic System Inc.) alone (d); coated with 10  $\mu$ g/ml (PBS) of the anti-CD3 antibody and 10  $\mu$ g/ml (PBS) of the anti-CD2 antibody TS2/18 (ATCC:HB195)(e); and coated with a mixture 10  $\mu$ g/ml (PBS) of the anti-CD3 antibody and 10  $\mu$ g/ml (PBS) of F(ab)<sub>2</sub> fragment of the anti-CD2 antibody TS2/18 at the same time (4°C, overnight)(f). The resulting plates were washed with PBS and therein was placed ~~each of~~ 100  $\mu$ l <sup>of 1.0  $\times$  10<sup>6</sup> cells/ml</sup> of 1.0  $\times$  10<sup>6</sup> cells/ml (less than the above mentioned cell number) of the normal human peripheral blood cell suspension and the culture was started. After the plates were subjected to culture at 37°C for 7 days in the 5 % CO<sub>2</sub> incubator, cells were recovered by pipetting and washed 3 times with PBS, and then the cells were suspended into RPMI-1640 including 10 % autologous serum to a final concentration of 3  $\times$  10<sup>6</sup> cells/ml.

#### COMPARATIVE EXAMPLE 1

25 Coating with cytokines and antibodies on usual culture devices and culture

The culture devices were coated in the same manner as

EXAMPLE 1 except that the usual 96-wells culture plates (made by Corning Inc.) were used. The cytokines and antibodies used in coating were washed out.

The human cells were seeded in the same manner as (a)  
5 of EXAMPLE 1 into non-coated 20 wells to give cell suspensions.

### TEST EXAMPLE 1

Suppression of activation of an antigen (PPD)-specific T  
cell by immunosuppressive cells obtained from human peripheral  
10 blood cells

Using RPMI-1640 (made by Sigma) including 10 %  
autologous serum,  $1 \times 10^6$  cells/ml of a normal human peripheral  
blood cell suspension was prepared from peripheral blood collected  
B from the normal human. ~~Each~~ of 100  $\mu$ l <sup>aliquots</sup> of the cell suspension  
B ~~was~~ <sup>were</sup> pipetted into a 96-wells round-bottom plate (made by Corning  
15 Inc.). PPD (made by Japan B.C.G Seizo Co., Ltd.) was placed in  
B each well to a final concentration of 1  $\mu$ g/ml. ~~Simultaneously~~ <sup>Simultaneously</sup>  
B ~~each~~ cells obtained in EXAMPLE 1 (a) to (f) were suspended in  
B RPMI-1640 including 10 % autologous serum, and ~~each~~ 30  $\mu$ l <sup>aliquots</sup> of  
20 the cell suspensions including  $0.25 \times 10^5$  cells/well and/or  $0.5 \times$   
B  $10^5$  cells/well <sup>were</sup> ~~was~~ placed in each ~~well~~ <sup>well</sup> and cultured for 5 days (in  
the 5 % CO<sub>2</sub> incubator, at 37 °C). After mixing 1  $\mu$  Ci of [<sup>3</sup>H]  
thymidine to each well and culturing for 15 hours, the cells were  
harvested using a cell harvester (LABO MASH; made by LABO  
25 SCIENCE). The radioactivity of <sup>3</sup>H incorporated in the cells was  
measured using the scintillation counter LSC-700 (made by Aloka).  
B As a positive control, in wells ~~was~~ <sup>were</sup> placed ~~each~~ 30  $\mu$ l of RPMI-1640

including 10 % autologous serum instead of the cells of EXAMPLE 1 and subjected to measurement.

The results are shown in Figures 3 to 6. When the cells treated in EXAMPLE 1(a) were mixed with one fourth or half of the number of the human cells applied to a PPD-T cell activating system, about 30 % suppression was observed (Figure 3). <sup>Where</sup>~~In case~~ that the cells treated in the presence of cytokines in EXAMPLE 1 (b) were used, about 55 % suppression was observed (Figure 4). The suppression was about 20 % when cells ~~of~~ treated in EXAMPLE 1 (c) were used (Figure 5), and when each of the cells treated in EXAMPLE 1 (d), (e) and (f) was used, about 50 %, 67 % and 78 % suppressions were observed, respectively (Figure 6).

The cells obtained in COMPARATIVE EXAMPLE 1 were suspended in the culture medium in the same manner as mentioned above and ~~each of~~ <sup>aliquots</sup> 30  $\mu$ l of the cell suspension including  $1.0 \times 10^5$  cells/well or  $0.5 \times 10^5$  cells/well <sup>were</sup> ~~was~~ placed in each ~~well~~ <sup>well</sup> and cultured as described above.

The result showed about -50 % and about -30 % suppressions, respectively. That is, when the human cells are cultured in the usual culture device, immunosuppressive cells were not induced, but rather the immunoreaction increased.

### REFERENCE EXAMPLE 2

#### Preparation of anti-human CD2 antibodies

Anti-human CD2 antibodies can be prepared by the following: (a) preparing CD2 protein from the human T cell line: Jurkat cell (ATCC TIB (Tumor Immunology Bank) 152); (b)

immunizing mice with the resulting CD2 protein to give hybridomas; and (c) screening hybridoma that can produce anti-CD2 antibodies by ELISA using the CD2 protein. The procedures thereof are as follows:

5

β (a) Preparation of the CD2 protein from a Jurkat cells

~~The Jurkat cell clones~~ <sup>Jurkat</sup>  $5.8 \times 10^8$  cells were cultured in 2500 ml of RPMI-1640 (made by Sigma) including 10 % FCS (Fetal Calf Serum) at 37°C, in the 5 % CO<sub>2</sub> incubator to a concentration of

10  $7 \times 10^5$  cells/ml and  $1.75 \times 10^9$  cells of the clones were subjected to

β purification. The ~~clones~~ <sup>clone</sup> were suspended in 40 ml of lysis buffer (0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1 % Triton X-100, 1 mM iodeacetoamide (made by Sigma), 1 mM PMSF), and treated with the mini disk rotor (made by Biocraft) at 4°C for 1 hour. Then,

15 β after centrifugation at  $3615 \times g$  at room temperature for 15 minutes, NaDOC (Sodium deoxycholate: made by Sigma) was mixed to a final concentration of 0.5 % NaDOC and supernatant was

β recovered by ultracentrifugation (at  $158,000 \times g$  4 °C, for 60 minutes). The resulting sample was subjected to purification using

20 the antibody column as a Jurkat-purified supernatant.

The antibody column was prepared using Affi-Gel 10 (made by Bio-Rad Laboratories, Inc.) as a carrier by immobilizing the anti-CD2 antibody TS2/18 (ATCC HB195) on the carrier (1 mg/ml gel). Econo-column(φ:1 cm, Height:10 cm, made by Bio-

25 Rad Laboratories, Inc.) was charged with 5 ml of immobilized gel.

The sample was loaded (1 ml/min), washed with 50 ml of PBS, followed by elution using 100 ml of elution buffer (0.1 M tri-

sodium citrate (pH3)). The sample eluted was brought to neutral pH by using the neutralization buffer (1 M Tris-HCl (pH11)).

The elution buffer was replaced by PBS with the dialysis cassette (made by Pierce), and the protein content in the sample was measured. As a result, it was confirmed that about 150  $\mu$ g of CD2 protein was obtained. Further, as the antigen for ~~immunization~~ <sup>immunization</sup> the ~~antigen~~ <sup>antigen purification</sup> purified with PEG 20,000 (made by Wako Pure Chemical Industries, Ltd.) was employed.

10 (b)immunization of mice by CD2 protein and isolation of hybridomas

The purified human CD2 obtained in the above (a) was mixed with a complete adjuvant Titer MAX (made by CytRX Corporation). The resulting mixture was peritoneally injected into ~~5-weeks age of~~ <sup>5-week old</sup> Balb/c mice (purchased by Japan SLC Co., Ltd.) a total of 4 times at an interval of a week (25  $\mu$ g/time/mouse). Antibodies raised in the mice immunized were subjected to titer confirmation by ELISA using the plate coated with CD2 protein (confirmation as to whether sufficient titer of antibodies were obtained), and the spleens were removed from the mice. A fifteen ~~ml of~~ <sup>B</sup> spleen cell suspension was prepared with IMDM (made by Sigma)/10 % FCS culture medium. Then, the suspension was centrifuged at room temperature at 1000 rpm for 10 minutes and cells were recovered. Ten ml of 0.17 M  $\text{NH}_4\text{Cl}$  was mixed with the cells for hemolysis. Again the suspension was centrifuged in the same manner as described above, and suspended into 10 ml of IMDM/10 % FCS culture medium to prepare a solution including 1

$\times 10^8$  cells of the spleen cells.

To prepare the hybridomas, the spleen cells and mouse myeloma cells SP2/O-Ag14 (ATCC CRL (Cell Repository Lines) 1581) were used. The cells were washed with IMDM culture medium and prepared to a final concentration of  $2 \times 10^7$  to  $3 \times 10^7$  cells.

The cells were fused by mixing the spleen cells and the myeloma cells at a ratio of 5 : 1 (spleen cells:  $1 \times 10^8$  cells and myeloma cells:  $2 \times 10^7$  cells), and the resulting fused cells were centrifuged at room temperature at 1000 rpm for 5 minutes, and then, <sup>recovered</sup> ~~recovered~~. After the cells were washed with 30 ml of IMDM culture medium, 2 ml of 50 % PEG 1500 (made by Boehringer Mannheim GmbH) was slowly mixed therewith, and with strongly shaking for 1 minute, 4 ml of IMDM culture medium was slowly mixed therewith for 2 minutes. Another 16 ml of IMDM culture medium was mixed therewith for 2 to 3 minutes, and again centrifuged in the same manner as mentioned above, and cells were recovered. Next, the resulting cells were suspended in 5 % briclone (made by Dainippon Pharmaceutical Co., Ltd.)-HAT (made by GIBCO BRL)-IMDM/10 % FCS culture medium to give a suspension of  $3 \times 10^6$  cells/ml. The cells were seeded in a 96-wells flat-bottom plate (made by IWAKI GLASS) at  $100 \mu$ l /well. After 7 days culture at  $37^\circ\text{C}$  in the 5 %  $\text{CO}_2$  incubator, the culture medium was exchanged to HT(made by GIBCO BRL)-IMDM/10 % FCS. The culture medium was changed every 3 days to isolate the hybridomas.

(c) Selection of anti-CD2 antibody-producing hybridomas

B — A 50  $\mu$ l of human CD2 solution (5  $\mu$ g/ml PBS) prepared  
 in the above-mentioned method (a) was placed in each ~~of wells~~<sup>well</sup> and  
 the 96-wells ELISA plate (made by Corning Inc.) ~~was coated~~<sup>and coating</sup> with  
 the solution<sup>occurred</sup> at 4°C overnight. After washing with PBS/0.05 %  
 Tween 20, a ~~blocking~~<sup>blocking</sup> treatment was performed using 4-times  
 diluted Blockace (made by Nakalai Tesque) for 2 hours at room  
 temperature. After washing with PBS/0.05 % Tween 20, ~~each~~  
 50  $\mu$ l<sup>aliquots</sup> of supernatant of the hybridomas ~~was~~<sup>were</sup> placed in each of ~~wells~~<sup>well</sup>,  
 and left for 1 hour at room temperature. After washing with  
 10 PBS/0.05 % Tween 20, 1000-times diluted anti-mouse IgG  
 antibody labeled with HRP (Horse Radish Peroxidase)(made by  
 Cappel) was mixed therewith, let stand for 1 hour at room  
 temperature, washed with PBS/0.05 % Tween 20, and 100  $\mu$ l of  
 chromophore liquid (10 mg of O-phenylenediamine tablet (made by  
 15 Sigma)<sup>9</sup> and 10  $\mu$ l of H<sub>2</sub>O<sub>2</sub> ~~were~~ dissolved in citrate-phosphate  
 buffer<sup>9</sup> was mixed therewith and reacted for 10 minutes. The  
 colored clones were selected as the anti-CD2 antibody-producing  
 hybridoma.

## EXAMPLE 2

20 Preparation of human peripheral blood cells cultured  
 with antibody-immobilized plastic plate

Among the hybridomas obtained in REFERENCE  
 EXAMPLE 2, seven clones with high reactivity in ELISA were  
 25 selected. The clones were cultured in serum-free medium at 37°C,  
 in the 5% CO<sub>2</sub> incubator for 4 days and thus culture media  
 including each antibodies were obtained. Then, each of those

antibody-including media and anti-CD2 antibody TS2/18 were  
 B coated on each of 20 <sup>well</sup> wells of a 96-<sup>well</sup> wells ELISA <sup>plate</sup> plates (made by  
 B Corning Inc.) (10  $\mu$ l of the culture medium of each <sup>clone</sup> clones and 0.5  $\mu$   
 g of TS2/18 were placed in each <sup>well</sup> wells and left at 4°C overnight).  
 B5 After washing with PBS, ~~each~~ <sup>100  $\mu$ l aliquots</sup> 100  $\mu$ l of normal human peripheral  
 blood cell suspension adjusted to a concentration of  $1.0 \times 10^6$   
 cells/ml using RPMI-1640 (made by Sigma) including 10 %  
 autologous serum were seeded to each wells. Similarly each 100  $\mu$   
 B 1 of the same cell suspension ~~was~~ <sup>were</sup> seeded into 20 wells coated with  
 10 0.5  $\mu$ g of TS2/18 alone and into 20 wells without coating. After  
 B incubation at 37°C in the 5 % CO<sub>2</sub> incubator for 7 days, ~~each of~~  
~~the cells in each 20 wells was~~ <sup>of the 20 wells were</sup> recovered and washed with PBS 3 times.  
 Then, the cells were suspended in RPMI-1640 culture medium  
 (made by Sigma) including 10 % autologous serum to a final  
 15 concentration of  $3 \times 10^6$  cells/ml.

## TEST EXAMPLE 2

Evaluation of the suppression ability of the cells treated  
 with the immobilized antibody on antigen (PPD)-specific T cell  
 20 activation reaction

B Into a 96-<sup>well</sup> wells round-bottom plate (made by Corning  
 B Inc.) ~~each~~ <sup>100  $\mu$ l aliquots</sup> 100  $\mu$ l of normal human peripheral blood cell  
 suspension adjusted to  $1.0 \times 10^6$  cells/ml using RPMI-1640 (made  
 by Sigma) including 10 % autologous serum ~~was~~ <sup>were</sup> poured. PPD  
 25 B (made by Japan B.C.G. Seizo Co., Ltd.) was placed in each ~~of wells~~ <sup>well</sup> wells  
 B to a final concentration of 1  $\mu$ g/ml. Simultaneously, ~~each~~ <sup>30  $\mu$ l aliquots</sup> 30  $\mu$ l of  
 cell suspensions including  $10^5$  cells/well of the cells prepared in



EXAMPLE 2 <sup>were</sup> ~~was~~ placed in the wells and cultured for 5 days (in the 5 % CO<sub>2</sub> incubator, at 37°C). Then, 1  $\mu$  Ci of [<sup>3</sup>H] thymidine was placed in each <sup>well</sup> ~~wells~~ cultured for 15 hours, and the cells were harvested using a cell harvester (LABO MASH, made by LABO SCIENCE). The radioactivity of <sup>3</sup>H incorporated in the cells was measured using the scintillation counter LSC-700 (made by Aloka).

As shown in <sup>Figure</sup> ~~Figure~~ 7, <sup>four</sup> ~~4~~ clones (S31A2NO1, S32D7NO9, S33H2NO10 and L12A9NO22) out of <sup>seven</sup> ~~7~~ clones were found to significantly suppress the PPD-T cell activation reaction when combined with TS2/18. The suppression rates thereof were S31A2NO1 (47 %), S32D7NO9(29 %), S33H2NO10(74 %) and L12A9NO22(34 %), respectively. Especially, the system employing the clone S33H2NO10 was observed to induce strongly immunosuppressive cells. The present experiment was performed in n=2, and the value in the Figures are averages of the experiments.

### INDUSTRIAL APPLICABILITY

According to the present invention, immunosuppressive cells can be induced efficiently by the immobilized culture. Therefore, the present invention can provide an efficient therapeutic system with fewer side-effects by which diseases with hypersensitivity of the immune system can be efficiently treated.